Oral Anti-CD3 Antibody Treatment Induces Regulatory T Cells and Inhibits the Development of Atherosclerosis in Mice

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Background—Accumulating evidence suggests that several subsets of regulatory T cells that actively mediate immunologic tolerance play crucial roles in atherogenesis. Recently, orally administered anti-CD3 monoclonal antibody has been shown as an inducer of novel regulatory T cells expressing latency-associated peptide (LAP) on their surface, which potently prevents systemic autoimmune responses. In the present study, we hypothesized that oral anti-CD3 antibody treatment may inhibit atherosclerosis in mice.

Methods and Results—Six-week-old apolipoprotein E–deficient mice on a standard diet were orally given anti-CD3 antibody or control immunoglobulin G on 5 consecutive days, and atherosclerosis was assessed at age 16 weeks. Oral administration of anti-CD3 antibody significantly reduced atherosclerotic lesion formation and accumulations of macrophages and CD4+ T cells in the plaques compared with controls. We observed a significant increase in LAP+ cells and CD25+Foxp3+ cells in the CD4+ T-cell population in anti-CD3–treated mice, in association with increased production of the antiinflammatory cytokine transforming growth factor–β and suppressed T-helper type 1 and type 2 immune responses. Neutralization of transforming growth factor–β in vivo abrogated the preventive effect of oral anti-CD3 antibody.

Conclusions—Our findings indicate the atheroprotective role of oral anti-CD3 antibody treatment in mice via induction of a regulatory T-cell response. These findings suggest that oral immune modulation may represent an attractive therapeutic approach to atherosclerosis. (Circulation. 2009;120:1996-2005.)

Key Words: atherosclerosis ■ immune system ■ inflammation ■ lymphocytes

Atherosclerosis is an inflammatory condition of the arterial wall involving cells of innate and adaptive immunity.1,2 It leads to severe clinical events caused by the rupture of plaques and thrombotic occlusion of the artery and is the main cause of acute coronary syndrome and stroke, which account for approximately half of the deaths in Western countries. It is now widely recognized that chronic inflammation via T-cell–mediated pathogenic immune responses plays an important role in atherogenesis.2 Accumulating evidence suggests that several subsets of regulatory T cells (Tregs), which have been shown to maintain immunologic unresponsiveness to self-antigens,3 inhibit atherosclerosis development through the downregulation of activated T-cell responses.4–6 These studies imply that promotion of an endogenous regulatory immune response has a therapeutic potential to suppress atherosclerotic diseases.

Clinical Perspective on p 2005

Anti-CD3–specific antibodies strongly suppress immune responses by antigenic modulation of the T-cell receptor/CD3 complex.7 In 1985, parenteral anti-CD3 antibody was available for use in acute transplant rejection in humans. However, its long-term use is limited because of severe side effects such as mitogenicity and antiglobulin responses.7 The mitogenicity of anti-CD3 antibody with Fc portion is caused by interacting with Fc receptors (FcRs) on monocytes or macrophages. Therefore, humanized Fc-mutated antibodies were designed and demonstrated to be effective in human autoimmune diabetes for a long period, although they still induce low levels of cytokine release because of some degree of T-cell activation.8 This long-term beneficial effect of FcR-nonbinding anti-CD3 antibody is partly explained by the recent study showing that FcR-nonbinding anti-CD3 antibody...
induces transforming growth factor (TGF)-β–producing CD4+CD25+ Tregs. Notably, it has been shown that intravenously administered FcR-nonbinding CD3-specific antibody induces a regulatory immune response and inhibits atherosclerosis development and progression. Recent studies have revealed that oral or nasal anti-CD3 antibody with or without Fc portion is biologically active and induces CD4+LAP+ (latency-associated peptide) Tregs that suppress experimental autoimmune encephalitis, autoimmune diabetes mellitus, and lupus in a TGF-β–dependent fashion and that this therapy would not be expected to have side effects even though FcR-binding CD3-specific antibody is used. Mucosal tolerance induction has been shown to inhibit various autoimmune diseases in mice and, importantly, atherosclerotic diseases in mice and therefore has been gaining much interest clinically. High doses of oral antigen lead to anergy or depletion of antigen-specific T cells. On the other hand, low doses of oral antigen induce many types of Tregs, including T-helper type 3 (Th3) cells, regulatory T-cell type 1 (Tr1) cells, naturally occurring CD4+CD25+Foxp3+ Tregs, and CD4+LAP+ Tregs, indicating that mucosal tolerance induction seems to be an effective way to treat atherosclerosis. As a possible explanation for the effects of oral anti-CD3 antibody treatment, a mechanism similar to that described in mucosal tolerance is supposed because autoimmune diseases are shown to be suppressed by low doses of oral anti-CD3 antibody but not by high doses. However, there is no evidence of antigen specificity with oral anti-CD3 antibody.

Given these backgrounds, we hypothesized that induction of CD4+LAP+ Tregs by oral anti-CD3 antibody treatment would inhibit atherosclerosis in apolipoprotein E–deficient (ApoE−/−) mice. Our findings provide for the first time evidence that oral anti-CD3 antibody treatment induces CD4+LAP+ Tregs and CD4+CD25+Foxp3+ Tregs, which suppress pathogenic immune processes pivotal for atherogenesis through a TGF-β–dependent mechanism and consequently inhibit atherosclerotic plaque formation.

Methods

For expanded Methods, please see the online-only Data Supplement.

Experimental Design

We fed 6-week-old or 16-week-old male ApoE−/− mice 5 μg hamster CD3-specific antibody (clone 145 to 2C11; R&D Systems, Minneapolis, Minn) or 5 μg hamster immunoglobulin G (IgG; Jackson Immuno Research Laboratories Inc, West Grove, Pa) dissolved in 0.2 mL phosphate-buffered saline by gastric intubation with a plastic tube once a day for 5 consecutive days. Mice were killed at 16 weeks of age, and atherosclerotic lesions were assessed. In some experiments, mice were fed control IgG or anti-CD3 antibody daily for 5 days and were injected with 100 μg of neutralizing anti-TGF-β antibody (clone 1D11; Bio Express Inc, West Lebanon, NH) or control rat IgG (Bio Express Inc). Injections were repeated once a week from age 7 weeks to age 16 weeks.

Atherosclerotic Lesion Assessment

For aortic root lesion analysis, 5 consecutive sections (10-μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse, stained with Oil Red O (Sigma, St Louis, Mo), and quantified with the use of the Image J (National Institutes of Health) as described previously. Immunohistochemistry was performed with the use of antibodies to identify macrophages (MOMA-2, 1:400; BMA Biomedicals, Augst, Switzerland), T cells (CD4, 1:100; BD Biosciences, San Jose, Calif), smooth muscle cells (α-smooth muscle actin, 1:400; Sigma), and natural Tregs (Foxp3, 1:100; eBioscience, San Diego, Calif). For en face lesion analysis, the thoracic aorta was opened longitudinally, stained with Oil Red O, and analyzed as described previously.

Flow Cytometry Analysis

Mesenteric lymph node (MLN) cells and splenocytes were isolated 2 days or 10 weeks after the final oral anti-CD3 antibody administration. Fluorescent-activated cell sorter analysis was performed by FACSComp with the use of CellQuest Pro software (BD Biosciences).

Cell Proliferation, Purification, and Cytokine Assays

Lymphocytes from MLNs or spleen were prepared and stimulated with concanavalin A (Sigma) in vitro. Cytokine levels in supernatants were examined by ELISA or a Mouse Cytokine Array kit (R&D Systems). CD4+CD25+LAP+ Tregs, CD4+CD25+LAP− cells, CD4+CD25− Tregs, and CD4+CD25− cells were purified from spleens or MLNs with the use of a MoFlo cell sorter (DakoCytomation, Denmark). Cell proliferation assay was performed by assessing [3H]thymidine incorporation.

Real-Time Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from MLN cells harvested after 2 days of oral antibody treatment or from the aortas after perfusion with RNA later (Ambion, Austin, Tex) with the use of TRizol reagent (Invitrogen, Carlsbad, Calif). Quantitative polymerase chain reaction (PCR) was performed as described previously.

Results

Effects of Oral Anti-CD3 Antibody on CD4+LAP+ Tregs or CD4+CD25+Foxp3+ Tregs in MLNs and Spleens

We fed 6-week-old male ApoE−/− mice 5 μg hamster anti-CD3–specific antibody or 5 μg hamster IgG by gastric intubation once a day for 5 consecutive days. Previous studies suggested a fundamental immunologic difference between intravenous and oral anti-CD3 antibody administration. In fact, oral anti-CD3 antibody treatment did not cause T-cell receptor downregulation or depletion of T cells (Figure 1 in the online-only Data Supplement). To investigate the effects of oral anti-CD3 antibody treatment on CD4+LAP+ Treg levels in ApoE−/− mice, we performed flow cytometry studies. Similar to the previous report, 2 days after the last feeding of anti-CD3 antibody, we found a significant increase in LAP+ Tregs in the CD4+ T-cell population in MLNs and spleens of anti-CD3–treated mice (P<0.05; Figure 1A and 1B). Notably, we observed a significant increase in LAP+ Tregs in the CD4+CD25+ T-cell population in spleens (P<0.05) but not in the CD4+CD25− T-cell population (Figure 1A and 1C), suggesting that oral anti-CD3 antibody treatment results in an increase in CD4+CD25+ LAP+ Tregs, which are distinct from naturally occurring Tregs expressing CD25+ on their surface. We also examined the effect of oral anti-CD3 treatment on natural CD4+CD25+Foxp3+ Tregs by fluorescence-activated cell sorter analyses. Interestingly, we found a modest but significant increase in CD4+CD25+Foxp3+ Tregs in MLNs of anti-CD3–treated mice compared with control mice (P<0.05) but not in spleens (Figure 1D and 1E).
To determine whether an increased number of Tregs by oral anti-CD3 contributes to the more suppressive potential of lymphoid cells, we performed in vitro proliferation assays of MLN cells or splenocytes in response to anti-CD3 antibody. As shown in Figure 1F and 1G, stimulation with anti-CD3 antibody resulted in the proliferation of MLN cells or splenocytes in control IgG-treated mice in a dose-dependent manner. In contrast, we observed a significant reduction in MLN or spleen cell proliferation of anti-CD3–treated mice (P<0.05), indicating the suppressed immune responses of lymphoid cells in anti-CD3–treated mice on polyclonal stimulation (Figure 1F and 1G). We additionally performed in vitro suppression assays to reveal whether oral anti-CD3 antibody treatment affects the suppressive function of Tregs.

These experiments demonstrated that the suppressive function of CD4⁺CD25⁺LAP⁺ Tregs or CD4⁺CD25⁺Foxp3⁺ Tregs was not altered by oral anti-CD3 treatment (Figure IIA and IIB in the online-only Data Supplement).

Oral Anti-CD3 Antibody Treatment Inhibits Atherosclerotic Plaque Formation and Reduces Inflammatory Cell Recruitment Into Plaques in ApoE⁻/⁻ Mice

To determine the effect of oral anti-CD3 treatment on the development of atherosclerosis, we orally treated 6-week-old male ApoE⁻/⁻ mice with a 5-μg dose of anti-CD3 antibody or control IgG daily for 5 consecutive days. During the experiments, no adverse effect was observed in either group. No
Table. Analysis of Plasma Lipid Profile and Body Weight at 16 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CD3-Ab</th>
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<tr>
<td>Mean±SEM</td>
<td>n</td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26.2±0.8</td>
<td>11</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>416.3±47.0</td>
<td>8</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>12.6±1.6</td>
<td>8</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>74.6±18.0</td>
<td>8</td>
</tr>
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HDL indicates high-density lipoprotein.

Statistical differences in body weight or plasma lipid profiles were detected between the 2 groups (Table). At 16 weeks of age, the mice were euthanized, and cryosections of the aortic root of control and anti-CD3–treated mice were stained with Oil Red O and analyzed quantitatively. Surprisingly, anti-CD3–treated mice showed ~50% reduction in atherosclerotic lesion formation in the aortic root compared with control IgG-treated mice (aortic sinus mean plaque area of 11 3854±20 329 μm² versus 54 565±5937 μm² in control IgG-treated and anti-CD3–treated mice, respectively; \( P = 0.007 \); Figure 2A). In parallel with the cross-sectional studies, en face analysis of thoracic aortas was performed. En face analysis of thoracic aortas revealed a significant 33% reduction in aortic plaque burden at 16 weeks in anti-CD3–treated mice (2.31±0.33% versus controls) compared with control mice (3.44±0.33%) (Figure 2B). To further evaluate the effect of anti-CD3 antibody treatment on the progression of atherosclerosis, we treated 16-week-old male ApoE<sup>−/−</sup> mice, euthanized them at 24 weeks, and evaluated the atherosclerotic lesion formation. The results indicated that oral anti-CD3 antibody treatment did not prevent atherosclerosis progression (Figure III in the online-only Data Supplement).

To determine the effects of oral anti-CD3 antibody treatment on plaque component, we next performed immunohistochemical studies of atherosclerotic lesions in the aortic sinus. Interestingly, the atherosclerotic lesions of anti-CD3–treated mice showed a marked 38% reduction in the accumulation of macrophages \( (P < 0.01) \) and also a 30% decrease in CD4<sup>+</sup> T-cell infiltration \( (P < 0.01) \) compared with control mice (Figure 3A and 3B). In addition, immunohistochemical analysis of the plaques showed that relative smooth muscle cell and collagen contents in the aortic sinus plaques tended to be increased in anti-CD3–treated mice, but this did not reach statistical significance (Figure 3A and 3B). These results suggest that oral anti-CD3 antibody treatment reduces plaque inflammation, which may lead to decreased lesion development.

Expression of Inflammatory Markers and Regulatory T-Cell Markers in Atherosclerotic Plaques

To reveal the mechanisms of reduced plaque development and inflammatory cell recruitment, we examined mRNA expressions of the proinflammatory molecules in atherosclerotic aortas by quantitative reverse transcription (RT)-PCR. We found that proinflammatory cytokines such as interferon (IFN)-γ and interleukin (IL)-6, adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, chemokine monocyte chemoattractant protein (MCP)-1, and macrophages detected by CD68 were markedly reduced in anti-CD3–treated mice compared with control mice \( (P < 0.05) \); Figure 4A and data not shown).

Recent studies suggest that antigen presentation may occur within the atherosclerotic plaque in addition to the lymphoid organs and that Tregs migrate to the atherosclerotic lesions to suppress local immune responses, although actual roles of intraplaque Tregs in atherogenesis have not yet been clarified. To determine whether increased accumulation of Tregs into the plaques is involved in the reduction of atherosclerotic lesion formation, we analyzed the expression of Treg-associated markers in the lesions by quantitative reverse transcription PCR (RT-PCR). We found no significant differences in the relative mRNA expressions of CD4<sup>+</sup> LAP<sup>+</sup> Treg-derived cytokine TGF-β or natural Treg-specific markers Foxp3 and CD25 in the plaques among the 2 groups (Figure 4A). Furthermore, immunohistochemical studies of

Figure 2. Oral anti-CD3 antibody administration inhibits atherosclerotic plaque formation. A, Representative photomicrographs of Oil Red O staining and quantitative analysis of atherosclerotic lesion size in the aortic sinus of male ApoE<sup>−/−</sup> mice treated with control IgG or anti-CD3 antibody. A black bar represents 200 μm. B, Representative photomicrographs of Oil Red O staining and quantitative analysis of atherosclerotic lesion size in the thoracic aortas of male ApoE<sup>−/−</sup> mice treated with control IgG or anti-CD3 antibody. Horizontal bars represent means. \( \ast P < 0.05 \) and \( \ast \ast P < 0.01 \) vs control IgG-treated mice.
atherosclerotic lesions with the use of anti-Foxp3 antibody demonstrated an increase in the number of natural Tregs within the plaque of anti-CD3–treated mice (Figure 4B). When it is taken into consideration that anti-CD3–treated mice have less plaque in the aorta than control mice, this result is consistent with the quantitative RT-PCR analysis of Treg-specific markers. These data indicate that oral anti-CD3 antibody treatment could inhibit the migration of effector T cells into the lesion selectively and relatively increase the proportion of natural Tregs in atherosclerotic plaque that might suppress pathogenic T-cell immune responses or macrophage activation as well as in the lymphoid organs.

Effects of Oral Anti-CD3 Antibody on Cytokine Production in Lymphocytes From Spleen and MLNs

Next, to determine whether induction of various Tregs by oral anti-CD3 antibody changed the cytokine production profile from lymphocytes, we examined cytokine secretion from spleen or MLN lymphocytes in response to concanavalin A using cytokine arrays and ELISA. On day 7 after the treatment of oral anti-CD3 antibody, we tried to assess the changes in cytokine production from MLN cells; however, the cytokine levels (IFN-γ, IL-4, and IL-10) were below the detectable levels. Therefore, we next investigated cytokine mRNA expression of unstimulated MLN cells by using quantitative RT-PCR. Interestingly, anti-CD3 antibody treatment resulted in a significant decrease in mRNA expressions of T-helper type 1 (Th1) cytokine (IFN-γ) and T-helper type 2 (Th2) cytokines (IL-4 and IL-10) in MLN cells (P<0.05; Figure 5A). Consistent with previous studies, significantly increased production of antiinflammatory cytokine TGF-β, which was the only detectable cytokine in our experiments, was observed in MLN cells of anti-CD3–treated mice (P<0.05; Figure 5B). CD4+ LAP+ Tregs induced by oral anti-CD3 antibody reach mesenteric lymph nodes and other
lymphoid organs such as spleens through the bloodstream, where they may induce deactivation of pathogenic T cells and macrophages. Therefore, we semiquantitatively analyzed various cytokine or chemokine productions in spleen lymphocytes on day 7 after the treatment. Lymphocytes from anti-CD3–treated mice secreted fewer Th1 cytokines (IFN-γ and IL-2), Th2 cytokines (IL-4, IL-10, and IL-13), and inflammation-related cytokines or chemokines (IL-17, MIP-1α, MIP-2, RANTES, and TNF-α) under stimulation with concanavalin A than those from control mice (Figure 5C), indicating suppressed Th1 and Th2 immune responses in anti-CD3–treated mice. ELISA data confirmed that TGF-β production was markedly increased and cytokine levels such as IFN-γ, IL-10, and IL-17 were significantly decreased in spleen lymphocyte supernatants from anti-CD3–treated mice compared with controls (P<0.05; Figure 5D). Although we could not detect IL-6 expression in the cytokine array analysis, ELISA data for lymphocyte IL-6 production revealed lower production in spleen lymphocytes of anti-CD3–treated mice than controls (P<0.05; Figure 5D). We also assessed cytokine production from lymphocytes 10 weeks after the treatment (at 16 weeks). Surprisingly, we found markedly increased TGF-β secretion in spleen lymphocytes of anti-CD3–treated mice (P<0.05), with no differences in IFN-γ or IL-10 levels between the groups (Figure 5E and data not shown), suggesting the long-term protective effect of TGF-β. In contrast to the increased TGF-β production at 16 weeks, we detected no significant difference in the number
of CD4⁺LAP⁺ Tregs among the 2 groups, although CD25⁺LAP⁺ Tregs in the CD4⁺ T cell population tended to be slightly increased in spleens of anti-CD3–treated mice (1.78±0.14% versus 2.11±0.15% in control IgG-treated and anti-CD3–treated mice, respectively; \( P=0.068 \); data not shown). Previous studies have shown that anti-CD3 antibody treatment not only increases the number of CD4⁺LAP⁺ Tregs but also enhances the regulatory function of these cells.11,12 Although we did not observe the enhanced suppressive function of Tregs in our in vivo experiments (Figure II in the online-only Data Supplement), our data imply that the prolonged TGF-\( \beta \) secretion in lymphocytes of anti-CD3–treated mice may reflect the altered function of these Tregs, which might be maintained for a long time, and suggest an important prolonged contribution of this antiinflammatory cytokine to a reduction in atherosclerosis development.

### Suppressive Function of Oral Anti-CD3 Antibody Is Mediated by TGF-\( \beta \)

Previous data suggest that TGF-\( \beta \) plays a key role in the suppressive function of both natural Tregs21 and CD4⁺LAP⁺ Tregs.22 In addition, our ELISA data for cytokine production in lymphocytes show that TGF-\( \beta \) is the only cytokine with an antiinflammatory property that is enhanced by anti-CD3 antibody treatment. To determine whether TGF-\( \beta \) is directly involved in preventing plaque formation after anti-CD3 treatment, we performed an in vivo TGF-\( \beta \) neutralization study. Anti-CD3–treated mice that received anti-TGF-\( \beta \) neutralizing antibody have significantly increased atherosclerotic lesion formation and accumulations of macrophages and CD4⁺ T cells in the plaques compared with anti-CD3–treated mice receiving isotype-matched antibody (Figure 6A and 6B). In addition, when mice were injected with neutralizing antibody, there were no significant differences in aortic root plaque size and inflammatory cell infiltration between control and anti-CD3–treated mice (Figure 6A and 6B). Together, these findings suggest that the suppressive function of oral anti-CD3 antibody in vivo is mediated by TGF-\( \beta \), at least in part.

### Discussion

Recent work has clearly demonstrated that natural Tregs, which show a high expression of CD25 on their surface23 and also express the transcription factor forkhead box P3 (Foxp3),24 play a protective role in atherogenesis in mice.4 Besides naturally occurring Tregs, previous studies have implicated the protective role of other types of Tregs including Tr1 or Th3 cells in the development of atherosclerosis in mice through the production of antiinflammatory cytokines such as IL-10 or TGF-\( \beta \), respectively.16,25 Recently, CD4⁺LAP⁺ Treg has been identified as a new subset of Tregs that suppresses autoimmune diseases in mice.11–13,22 In the present study, we show that oral anti-CD3 antibody treatment attenuates atherosclerotic lesion formation and that this reduction is associated with an increase in the number of Tregs, including CD4⁺LAP⁺ Tregs and CD4⁺CD25⁺Foxp3⁺ Tregs, which are possibly induced in the gut and migrate into other lymphoid organs, subsequently leading to suppression of Th1 and Th2 immune responses through the production of TGF-\( \beta \). Our data are the first report demonstrating a possible role of CD4⁺LAP⁺ Tregs in atherogenesis, although further studies are required to provide more direct evidence for the relationship between an enhancement in CD4⁺LAP⁺ Treg number or function and a reduction of atherosclerosis.

Importantly, unlike the case of intravenous FcR-binding anti-CD3 antibody treatment, we observed no evidence of adverse responses of oral FcR-binding anti-CD3 antibody during the experiment, which may be related to the fact that orally administered anti-CD3 antibody is very small in amount (10 times less than that of the usual intravenous...
administration) and for the most part does not seem to enter the bloodstream.\(^\text{11}\) It has been reported that immunosuppressive effects by intravenous FcR-binding anti-CD3 antibody may involve mechanisms such as depletion of pathogenetic T cells from the bloodstream or lymphoid organs and modulation of the T-cell receptor on T cells.\(^\text{7}\) In contrast to intravenous FcR-binding anti-CD3 antibody administration, we never observed the T-cell receptor downmodulation or depletion of T cells (Figure I in the online-only Data Supplement), which is consistent with the previous report.\(^\text{11}\) Ochi et al\(^\text{11}\) speculated that low doses of oral anti-CD3 antibody administration may result in the induction of Tregs by delivering a weak signal to T cells, which is observed similarly in mucosal tolerance induction. They demonstrated that experimental autoimmune encephalitis was suppressed in mice fed 5 \(\mu\)g anti-CD3 antibody with or without Fc portion but not in mice fed 50 \(\mu\)g or 500 \(\mu\)g antibody.\(^\text{11}\) Consistent with its effect on suppression of experimental autoimmune encephalitis, it was also shown that suppression of proliferation in spleens and MLN cells was observed with 5 \(\mu\)g anti-CD3 antibody but not with 50 \(\mu\)g or 500 \(\mu\)g. In consideration of this, we decided to use a 5-\(\mu\)g dose of FcR-binding anti-CD3 antibody for the treatment of 

\text{ApoE}\(^\text{-/-}\) mice and found that this treatment results in a dramatic reduction in atherosclerotic lesion formation, associated with a significant increase in CD4\(^+\) LAP\(^+\) Tregs. Intriguingly, we also detected an increased proportion of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs in MLNs of anti-CD3–treated mice. Administration of FcR-nonbinding anti-CD3 monoclonal antibody has been shown to induce long-lasting immune tolerance and to be an effective treatment for autoimmune diabetes in mice and in humans,\(^\text{8,9}\) acute transplant rejection in humans,\(^\text{7}\) and, notably, atherosclerosis in mice.\(^\text{10}\) These studies have indicated that the long-term beneficial effect of FcR-nonbinding anti-CD3 antibody may possibly be caused by increasing TGF-\(\beta\)-producing Tregs. When parenteral FcR-nonbinding anti-CD3 antibody is compared with oral FcR-binding anti-CD3 antibody, there may be some common mechanisms underlying suppression of autoimmunity because both appear to work mainly by inducing TGF-\(\beta\)-producing Tregs rather than eliminating pathogenetic T cells. In consideration of this, it is possible that after oral administration, FcR-binding anti-CD3 antibody might lose its Fc portion in the gut and consequently produce F(ab)\(^{\prime}\)\(_2\) (FcR-nonbinding anti-CD3 antibody), a small amount of which might enter the bloodstream, leading to induction of Tregs.

Several in vitro and in vivo studies demonstrated that naive T cells in the periphery can differentiate into CD4\(^+\)Foxp3\(^+\) Tregs in the presence of TGF-\(\beta\).\(^\text{26,27}\) Recent studies also suggest that differentiation of CD4\(^+\)Foxp3\(^+\) Tregs may be facilitated by dendritic cells in the gut-associated lymphoid tissue in the presence of TGF-\(\beta\) and that retinoic acid produced by dendritic cells plays an important role in their differentiation.\(^\text{28}\) In this study, we found a significant increase in CD4\(^+\) LAP\(^+\) Tregs in MLNs and spleens of anti-CD3–treated mice, in association with markedly increased TGF-\(\beta\) secretion by CD4\(^+\) LAP\(^+\) Tregs, which have been reported to secrete much more TGF-\(\beta\) than CD4\(^+\) LAP T cells.\(^\text{11}\) Therefore, it is highly possible that upregulated TGF-\(\beta\) secretion in the gut from anti-CD3–induced CD4\(^+\) LAP\(^+\) Tregs could lead to induction of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs.

Because previous studies have reported that oral anti-CD3 treatment results in conversion of Th1 immune responses into Th2 or Th3 immune responses in the periphery associated with decreased proliferation in spleen cells and MLN cells,\(^\text{31,32}\) it seems possible that similar changes in the cytokine production profile from lymphocytes could contribute to the observed antiatherogenic effect of oral anti-CD3 antibody in our study. Unlike previous studies, however, our ELISA and cytokine array analyses clearly demonstrated significant suppression in both Th1 and Th2 immune responses, as well as markedly increased TGF-\(\beta\) production from lymphocytes in oral anti-CD3–treated mice. In this experiment, we used atherosclerosis-prone ApoE\(^\text{-/-}\) mice on C57BL/6 background, which are known to show Th1-prone phenotype. Therefore, the difference in mouse genetic background used might be a possible explanation for the distinct immune responses by oral anti-CD3 antibody between our study and others. Accumulating evidence suggests that T-helper 17 (Th17) cells, which produce the inflammatory cytokine IL-17, play an important role in promotion of many autoimmune diseases.\(^\text{29}\) In addition, it has been reported that TGF-\(\beta\) stimulation leads to differentiation of naive T cells to Th17 cells or CD4\(^+\) Foxp3\(^+\) Tregs in mice in the presence or absence of IL-6, respectively.\(^\text{30}\) Our data indicate a shift from Th17 cells to CD4\(^+\) Foxp3\(^+\) Tregs in oral anti-CD3–treated mice, which might partially affect the atheroprotective effects of oral anti-CD3, although whether Th17 cells accelerate atherosclerosis as well as other autoimmune diseases thus far remains unclear.

TGF-\(\beta\) has been attracting much attention as a potent antiatherosclerotic cytokine.\(^\text{31}\) TGF-\(\beta\), which is produced by many types of cells in atherosclerotic plaques, suppresses pathogenic immune responses such as recruitment of inflammatory cells into the plaques or foam cell formation and increases collagen biosynthesis.\(^\text{32,33}\) In particular, TGF-\(\beta\) signaling in T cells appears to be indispensable for the regulation of atherosclerotic disease progression because ApoE\(^\text{-/-}\) mice with specific deletion of TGF-\(\beta\) signaling in T cells show remarkably accelerated plaque formation and inflammatory cell infiltration, associated with enhancement of both Th1 and Th2 immune responses.\(^\text{34}\) In consideration of this, our data suggest that increased TGF-\(\beta\) production from T cells may be a key mechanism for the preventive effect of oral anti-CD3 antibody on atherosclerosis through the downregulation in both Th1 and Th2 immune responses.

We are somewhat surprised because only 5-day oral treatment with a small amount of anti-CD3 antibody has a prolonged effect on the immune system and results in a dramatic atheroprotective phenotype. This long-term protective effect of oral anti-CD3 antibody was observed similarly in other autoimmune diseases such as experimental autoimmune encephalitis and autoimmune diabetes.\(^\text{11,12}\) TGF-\(\beta\) appears to play a crucial role in the prolonged antiatherogenic effect because we detected a significant increase in TGF-\(\beta\) production in spleen lymphocytes of oral anti-CD3–treated mice at 10 weeks after the antibody administration. It is reported that CD4\(^+\) LAP\(^+\) Tregs secrete TGF-\(\beta\) and are also
activated by TGF-β produced by themselves, indicating that they may act in autocrine and paracrine manners. We speculate that a transient increase in CD4+ LAP+ Tregs may lead to a positive-feedback loop of activation of CD4+ LAP+ Tregs through the production of TGF-β. On the basis of our observed findings, this novel method, which directly governs the inflammatory immune responses in atherogenesis, could be a hopeful strategy to prevent cardiovascular diseases.

In summary, we have demonstrated that oral anti-CD3 antibody treatment inhibits atherosclerosis development via inducing various Tregs and that TGF-β, which may be derived mainly from CD4+ LAP+ Tregs, possibly plays an important role in this suppressive mechanism. Although the mechanism by which oral anti-CD3 antibody increases Tregs remains to be clarified, our data indicate that induction of Tregs in the intestine by oral anti-CD3 antibody could be used as a promising therapeutic approach for atherosclerotic vascular disorders. Recent studies have demonstrated no toxicity of orally administered anti-CD3 antibody not only in mice but also in humans. Clinical studies in human are required to identify the efficacy of oral anti-CD3 antibody in the prevention of atherosclerotic diseases.

Acknowledgments
We thank Tomoyuki Yamaguchi for helpful suggestions and Michiyo Nakata for technical assistance.

Sources of Funding
This work was supported by a Grant-in-Aid for Scientific Research in Japan and by research grants from the Jinsenkai Medical Foundation, Kanae Medical Foundation, Suzuken Memorial Foundation, and Uehara Medical Foundation.

Disclosures
None.

References
CLINICAL PERSPECTIVE

Atherosclerosis is a chronic inflammatory condition of the arterial wall involving immune cells and their releasing molecules. This important notion has led to a quest to develop an antiinflammatory and immune therapy for prevention of atherosclerosis in clinical patients. Accumulating evidence suggests that several subsets of regulatory T cells (Tregs) inhibit atherosclerosis development through the downregulation of activated T-cell responses. Recent studies have demonstrated that parenteral administration of anti-CD3–specific antibody restores long-lasting self-tolerance and is effective in autoimmune diabetes in mice and humans, partly because of the expansion of CD4⁺CD25⁺ Tregs. Interestingly, orally administered anti-CD3 antibody has been shown to prevent systemic autoimmunity through induction of novel Tregs expressing latency-associated peptide (LAP) on their surface in the gut. In the present study, we first show that oral anti-CD3 antibody treatment attenuates atherosclerotic lesion formation and plaque inflammation and that this suppression is associated with an increase in the number of CD4⁺CD25⁺LAP⁺ Tregs and, unexpectedly, CD4⁺CD25⁺Foxp3⁺ Tregs, which are possibly induced in the gut-associated lymphoid tissue and migrate into other lymphoid organs or atherosclerotic plaques, subsequently leading to suppression of T-helper type 1 and type 2 immune responses through a transforming growth factor-β–dependent mechanism. Our data indicate that oral administration of anti-CD3 antibody seems to be safe and easy to apply and therefore could be used as a promising therapeutic approach for atherosclerotic vascular disorders. Clinical studies are required.
Oral Anti-CD3 Antibody Treatment Induces Regulatory T Cells and Inhibits the Development of Atherosclerosis in Mice
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Circulation. 2009;120:1996-2005; originally published online November 2, 2009;
doi: 10.1161/CIRCULATIONAHA.109.863431
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/120/20/1996

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Oral Anti-CD3 Antibody Treatment Induces Regulatory T Cells and Ameliorates the Development of Atherosclerosis in Mice

Naoto Sasaki, Tomoya Yamashita, Masafumi Takeda, Masakazu Shinohara, Kenji Nakajima, Hideto Tawa, Takashi Usui, Ken-ichi Hirata

Supplemental Methods

Animals

We housed mice in a specific pathogen-free animal facility at Kobe University, and all animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine. Male ApoE/− mice (offspring of homozygous ApoE-KO mice, backcrossed onto the C57BL/6 background) were fed a normal chow (Oriental Yeast, Tokyo, Japan) and water ad libitum and were maintained on a 12:12-h light-dark cycle in the animal facility.

Experimental Design

We fed 6-week-old male ApoE/− mice 5μg hamster CD3-specific antibody (clone 145-2C11; R&D Systems, Minneapolis, MN) or 5μg hamster IgG (Jackson Immuno Research Laboratories Inc, West Grove, PA) dissolved in 0.2 mL PBS by gastric intubation with a plastic tube once a day for five consecutive days. Mice were killed at
16 weeks of age, and atherosclerotic lesions were assessed. To further evaluate the effect of anti-CD3 antibody treatment on the progression of atherosclerosis, 16-week-old male ApoE−/− mice were treated with anti-CD3 antibody or control hamster IgG as described above, killed at 24 weeks of age, and atherosclerotic lesions were assessed. In some experiments, mice were fed control IgG or anti-CD3 antibody daily for 5 days, and were injected with 100μg of neutralizing anti-TGF-β antibody (clone 1D11; Bio Express Inc, West Lebanon, NH) or control rat IgG (Bio Express Inc). Injections were repeated once a week from 7-week-old to 16-week-old.

Blood Analysis

After overnight fasting, blood was collected by the cardiac puncture under anesthesia using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation and stored at -80°C. Concentrations of plasma total cholesterol, high density lipoprotein and triglyceride were determined enzymatically using an automated chemistry analyzer (SRL, Tokyo, Japan).

Atherosclerotic Lesion Assessment

At 16 or 24 weeks of age, mice were anesthetized and the aorta was perfused with saline. The aorta was dissected from the middle of the left ventricle to the bifurcation of the iliac artery. For aortic root lesion analysis, the samples were cut in the ascending aorta, and the proximal samples containing the aortic sinus were embedded in OCT compounds (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Five consecutive sections (10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and stained with Oil Red O (Sigma, St Louis, MO). For quantitative analysis of
atherosclerosis, the total lesion area of 5 separate sections from each mouse was obtained with the use of the Image J (National Institutes of Health) as previously described.\textsuperscript{1} For en face lesion analysis, the aorta was excised from the proximal ascending aorta to the thoracic aorta and fixed in 10% buffered formalin. After the adventitial tissue was carefully removed, the aorta was opened longitudinally, stained with Oil Red O, pinned on a black wax surface, and captured digitally with a digital camera. The percentage of stained lesion area per total area of the aorta was determined by Image J as described previously.\textsuperscript{1} Lesion analysis was conducted by a single observer blinded to the group of the mice.

**Immunohistochemical Analysis of Atherosclerotic Lesions**

Immunohistochemistry was performed on acetone-fixed or formalin-fixed cryosections (10\(\mu\)m) of mouse aortic roots using antibodies to identify macrophages (MOMA-2, 1:400; BMA Biomedicals, Augst, Switzerland), and T cells (CD4, 1:100; BD Biosciences, San Jose, CA), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Acetone-fixed cryosections of mouse aortic roots were incubated with rat anti-Foxp3 antibody (clone FJK-16s, 1:100; eBioscience, San Diego, CA), followed by Alexa Fluor 588 anti-rat secondary antibody (1:500; Molecular Probes, Eugene, OR) and examined using a LSM5 PASCAL laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Nuclei were counterstained with TOPRO-3 (Molecular Probes). Smooth muscle cells were identified by immunostaining with fluorescein isothiocyanate (FITC)-conjugated primary antibody against \(\alpha\)-smooth muscle actin (clone 1A4, 1:400; Sigma), followed by anti-FITC biotin-conjugated secondary antibody (1:400; Sigma). The appropriate
fixation reagent depending on the primary antibodies was used. Negative controls were prepared with substitution with an isotype control antibody. Staining with Masson’s trichrome was used to delineate the fibrous area as previously described.\textsuperscript{2} Stained sections were digitally captured, and the percentage of the stained area (the stained area per total atherosclerotic lesion area) was calculated. Quantification of CD4-positive T cells or Foxp3-positive cells was done by counting positively stained cells, which was divided by total plaque area.

**Flow Cytometry Analysis**

For fluorescent-activated cell sorter (FACS) analysis of lymphoid organs, mesenteric lymph node (MLN) cells and splenocytes were isolated 2 days or 10 weeks after the final oral anti-CD3 antibody administration. Cells were stained in PBS containing 2% FCS. FACS analysis was performed by FACSCalibur using CellQuest Pro software (BD Bioscience). The antibodies used were as follows; FITC-conjugated anti-CD3 (clone 145-2C11; BD Bioscience), PE-conjugated anti-TCR\(\beta\) (clone H57-597; BD Bioscience), FITC-conjugated anti-CD4 (clone H129.19; BD Bioscience), phycoerythrin (PE)-conjugated anti-CD25 (clone PC61; BD Bioscience), allophycocyanin (APC)-conjugated Foxp3 (clone FJK-16s; eBioscience), APC-labeled streptavidin, biotinylated anti-human LAP (BAF246; R&D Systems), and isotype matched control antibodies. For LAP staining, single cell suspension was first stained using biotinylated anti-LAP antibody, followed by other antibodies including APC-labeled streptavidin as described previously.\textsuperscript{3} Intracellular staining of Foxp3 was performed using the Foxp3 staining buffer set (eBioscience) according to the manufacturer’s instructions. All staining procedure was performed after blocking Fc
receptor with anti-CD16/CD32 (clone 2.4G2; BD Bioscience). Surface stainings were performed according to standard procedures at a density of $1 \times 10^6$ cells per 100μl, and volumes were scaled up accordingly.

Cell Proliferation, Purification, and Cytokine Assays

In all cell culture experiments, we used RPMI 1640 medium (Sigma) supplemented with 10% FCS, 10mmol/L Hepes, 50μmol/L 2β-mercaptoethanol and antibiotics. Mononuclear cells were isolated from spleen using Ficoll-Paque (GE Healthcare). After washing by PBS with 2% FCS, these separated cells were plated in culture dishes in RPMI medium for 2 hours at 37 °C. The non-adhesive cells were collected and cultured in 24-well plates in RPMI medium at a concentration of $1 \times 10^6$ cells/mL and stimulated with 2 μg/mL concanavalin A (Con A; Sigma). For the culture of mesenteric lymph node cells, whole isolated cells were used. Culture supernatants were collected at 72 hours and analyzed by ELISA for interleukin (IL)-6, IL-10, IL-17 and interferon (IFN)-γ using paired antibodies specific for corresponding cytokines according to the manufacturer's instructions (R & D Systems). Semiquantitative determination of multiple inflammation-associated cytokines and chemokines in cell culture supernates was performed using a Mouse Cytokine Array kit according to the manufacturer's instructions (R & D Systems). For TGF-β assay, the cells were cultured in serum-free RPMI medium under the same condition as the other cytokines described above, and culture supernatants were collected at 72 hours. The cell supernatants were acidified by addition of HCl, neutralized by NaOH, and TGF-β production was determined using paired antibodies specific for TGF-β (R & D Systems) according to the manufacturer’s instruction. For the cell proliferation assay,
splenocytes or mesenteric lymph node cells were cultured at 0.2x10^6 cells/well (total volume, 200μL/well) in flat-bottomed 96-well plates and stimulated with purified soluble CD3-specific antibody (1μg/mL, R & D Systems) at 37°C with 5% CO₂ for 72 hours. For analysis of in vitro suppressive function of CD4⁺CD25⁺LAP⁺ Tregs, CD4⁺CD25⁺LAP⁺ Tregs and CD4⁺CD25⁺LAP⁻ cells were purified from spleens using a MoFlo cell sorter (DakoCytomation, Denmark). The purity of each population was >90% by FACS analysis. Purified CD4⁺CD25⁺LAP⁺ Tregs from control IgG or anti-CD3-treated mice were co-cultured with responder CD4⁺CD25⁺LAP⁻ cells (5x10^3 cell per well) at the indicated ratios in the presence of soluble anti-CD3 antibody (1μg/mL, clone 145-2C11; BD Bioscience). For analysis of in vitro suppressive function of CD4⁺CD25⁺ Tregs, CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ cells were purified from MLNs using a MoFlo cell sorter (DakoCytomation). The purity of each population was >98% by FACS analysis. Purified CD4⁺CD25⁺ Tregs from control IgG or anti-CD3-treated mice were co-cultured with responder CD4⁺CD25⁻ cells (2.5x10^5 cell per well) at the indicated ratios in the presence of irradiated APCs (5x10^5 cell per well) and soluble anti-CD3 antibody (0.5μg/mL, BD Bioscience). The co-cultured cells were maintained at 37°C with 5% CO₂ for 4 days. The cells were pulsed with 1 μCi of [³H]-thymidine for the last 12 to 16 hours, and thymidine incorporation was assessed with a LS 6500 liquid scintillation counter (Beckman Coulter Inc) (mean±SEM of triplicate wells).

**Real-Time RT-PCR Analysis**

At 16 weeks old, mice were anesthetized and the whole aortas were excised as described above. Total RNA was extracted from MLN cells harvested after 2days of
oral antibody treatment or from the aortas after perfusion with RNA later (Ambion, Austin, TX) using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using One Step SYBR PrimeScript RT-PCR Kit (Takara, Shiga, Japan) and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The following primers were used to amplify intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, monocyte chemoattractant protein (MCP)-1, IFN-γ, IL-4, IL-6, IL-10, TGF-β, CD68, CD25, Foxp3 and GAPDH: ICAM-1, 5’-CAA TTC ACA CTG AAT GCC AGC TC-3’ and 5’-CAA GCA AGT CCG TCT CGT CCA-3’; VCAM-1, 5’-TGC CGG CAT ATA CGA GTG TGA-3’ and 5’-CCC GAT GGC AGG TAT TAC CAA G-3’; MCP-1, 5’-GCA TCC ACG TGT TGG CTC A-3’ and 5’-CTC CAG CCT ACT CAT TGG GAT GAG TCT GA-3’; IFN-γ, 5’-CGG CAC AGT CAT TGA AAG CC T A-3’ and 5’-GTT GCT GAT GGC CTG ATT GTC-3’; IL-4, 5’-TCT CGA ATG TAC CAG GAG CCA TAT C-3’ and 5’-AGC ACC TTG GAA GCC CTA CAG A-3’; IL-6, 5’-CCA CTT CAC AAG TCG GAG TCT GA-3’ and 5’-GAG GCT TTG AAT GTG GCA TTG-3’; IL-10, 5’-GAC CAG CTG GAC AAC ATA CTG CTA A-3’ and 5’-GAT AAG GCT TGG CAA CCC AAG TAA-3’; TGF-β, 5’-GTG TGG AGC AAC ATG TGG AAC TCT A-3’ and 5’-TTG GTT CAG CCT AGT CTA TGG TGG CAG GCT TA-3’; CD68, 5’-CAT CAG AGC CCG AGT ACA GTC TAC C-3’ and 5’-AAT TCT GCG CCA TGA ATG TCC-3’; CD25, 5’-CTG ATC CCA TGT GCC AGG AA-3’ and 5’-AGG GCT TTG GAT GTG GCC TTG-3’; Foxp3, 5’-CTC ATG ATA GTG CCT GTG TCC TCA A-3’ and 5’-AGGG GCC AGC ATA GGT GCA AG-3’; GAPDH, 5’-TGT GTC CTG CGT GGA TCT GA-3’ and 5’-TTG CTG TTG AAG TCG CAG GAG-3’. Amplification reactions were performed in duplicate and fluorescence curves were analyzed with included software. GAPDH was used as
an endogenous control reference.

**Statistical Analysis**

Data were expressed as means±SEM. Mann-Whitney *U* test was used to detect significant differences between the 2 groups. Kruskal-Wallis test was used to detect significant differences when 3 groups were compared in *in vivo* TGF-β neutralization study. Statistical Values of *P*<0.05 were considered statistically significant. For statistical analysis, GraphPad Prism version 4.0 (GraphPad Software, San Diego, Calif) was used.

**Supplemental References**


**Supplemental Figure Legends**

**Supplemental Figure I.**
Effects of oral anti-CD3 antibody administration on T-cell receptor down-modulation or depletion of T cells in blood cells and spleens. *ApoE<sup>−/−</sup>* mice were fed control IgG or anti-CD3 antibody daily for 5 days. Two days after the last feeding, lymphoid cells from blood and spleen were prepared and stained with FITC-anti-CD3, and PE-anti-TCRβ. Blood lymphoid cells were collected from 5 mice in each group and analyzed by FACS. The graphs represent the percentage of CD3<sup>+</sup> cells or TCRβ<sup>+</sup> cells within the blood cells (A) and splenocytes (n=5 per group) (B).

**Supplemental Figure II.**
Oral anti-CD3 antibody dose not affect regulatory function of CD4<sup>+</sup>CD25<sup>−</sup>LAP<sup>+</sup> Tregs (A) and CD4<sup>+</sup>CD25<sup>+</sup> Tregs (B). A, Two days after the final oral antibody administration, CD4<sup>+</sup>CD25<sup>−</sup>LAP<sup>+</sup> Tregs and CD4<sup>+</sup>CD25<sup>−</sup>LAP<sup>−</sup> cells were purified from spleens by FACS sorting. CD4<sup>+</sup>CD25<sup>−</sup>LAP<sup>+</sup> Tregs from control IgG or anti-CD3-treated mice were co-cultured with responder CD4<sup>+</sup>CD25<sup>−</sup>LAP<sup>−</sup> cells (5×10<sup>3</sup> cell per well) at the indicated ratios in the presence of soluble anti-CD3 antibody (1μg/ml). B, Two days after the final oral antibody administration, CD4<sup>+</sup>CD25<sup>+</sup> Tregs and CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from MLNs by FACS sorting. CD4<sup>+</sup>CD25<sup>+</sup>Tregs from control IgG or anti-CD3-treated mice were co-cultured with responder CD4<sup>+</sup>CD25<sup>−</sup> cells (2.5×10<sup>5</sup> cell per well) at the indicated ratios in the presence of irradiated APCs (5×10<sup>5</sup> cell per well) and soluble anti-CD3 antibody (0.5μg/ml).
Proliferation of these cells were assessed by $[^3]$H-thymidine incorporation (mean±SEM of triplicate wells).

**Supplemental Figure III.**

Oral anti-CD3 antibody administration does not inhibit atherosclerotic plaque progression. Sixteen-week-old male $ApoE^{-/-}$ mice were orally treated with anti-CD3 antibody or control IgG, killed at 24 weeks of age, and atherosclerotic lesions were assessed. A, Representative photomicrographs of Oil Red O staining and quantitative analysis of atherosclerotic lesion size in the aortic sinus. A black bar represents 200 μm. B, Representative photomicrographs of Oil Red O staining and quantitative analysis of atherosclerotic lesion size in the thoracic aortas. Horizontal bars represent means.
Supplemental Figure I.
Supplemental Figure II.
Supplemental Figure III.